INTERACTIONS BETWEEN pH AND MALIC ACID CONCENTRATION ON THE INACTIVATION OF *LISTERIA MONOCYTOGENES*[1]

R.L. BUCHANAN[2] and M.H. GOLDEN

U.S. Department of Agriculture
Agricultural Research Service
Eastern Regional Research Center
600 East Mermaid Lane
Wyndmoor, PA 19038

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ABSTRACT

The effects and interactions of malic acid concentration and pH on the inactivation kinetics of a three strain mixture of Listeria monocytogenes *was studied in brain heart infusion broth (BHI). The medium was supplemented with malic acid and monosodium malate to achieve pH levels of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 or 7.0 in conjunction with concentrations of 0.0, 0.1, 0.5, 1.0 and 2.0 M. Duplicate 20-mL portions of each pH/malate level were inoculated (ca. 10^8 cfu/mL), stored aerobically at 28C, and assayed periodically for viable counts by plating on BHI agar. Survivor curves were generated by fitting data to a linear model that includes a lag term and used to calculate D-values and "times to 4-D inactivation". Inactivation rates were dependent on both the pH and malic acid concentration. At the higher pH levels, malic acid appeared to provide some degree of protection compared to control cultures where the pH was adjusted with HCl. At lower pH values and at higher malic acid concentrations, a concentration-dependent anion effect was observed. The results indicate that malic acid is a relatively benign organic acid. Its antimicrobial characteristics are similar to those of citric acid and is substantially less bactericidal than lactic or acetic acids.*

[1]Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

INTRODUCTION

Controlling foodborne *Listeria monocytogenes* has become major concern to manufacturers of refrigerated ready-to-eat foods, especially those with extended shelf lives, due to the pathogen's high mortality rates in susceptible populations and it's abilities to grow at refrigerated temperatures and tolerate acidic conditions (Buchanan and Klawitter 1990; Conner *et al.* 1990; Farber *et al.* 1989; Ita and Hutkins 1991; Walker *et al.* 1990). Acidification with short-chain organic acids by either fermentation or direct addition is the major means of controlling foodborne pathogens in a wide range of ready-to-eat foods. However, certain bacteria such as *L. monocytogenes* and enterohemorrhagic *Escherichia coli* have the ability to survive acidic conditions for extended periods, particularly at refrigerated temperatures (Buchanan *et al.* 1994; Conner and Kotrola 1995; Leyer *et al.* 1995). Further, their acid tolerance can be enhanced by pre-exposing the microorganisms to moderately acidic conditions (Kroll and Patchett 1992; Buchanan *et al.* 1994; Small *et al.* 1994; Leyer *et al.* 1995; Buchanan and Edelson 1996).

Acids vary greatly in both their ability to inhibit growth of *L. monocytogenes* and to accelerate its inactivation under acidic conditions (Ahamad and Marth 1989; Cherrington *et al.* 1992; Conner *et al.* 1990; Ita and Hutkins 1991; Kouassi and Shelef 1996; Sorrels *et al.* 1989; Young and Foegeding 1993). Previously, we studied the effects and interactions of pH and acetic, lactic, and citric acid concentrations on the kinetics of nonthermal inactivation of *L. monocytogenes* (Buchanan and Golden 1994; Buchanan *et al.* 1993; 1994; 1997). The association of recent outbreaks of hemorrhagic colitis and other foodborne diseases to unpasteurized apple cider, a traditional product that relies on its acidic pH (ca. 3.5-4.0) to control foodborne pathogens, has increased interest in the antimicrobial activity of malic acid. Malic acid, a four carbon dicarboxylic acid, is the primary organic acid in apples (Hulme and Rhodes 1971). *L. monocytogenes* has been reported to grow at pH levels as low as 4.4 in the presence of malic acid (Sorrells *et al.* 1989). Similarly, Glass *et al.* (1995) found that malic acid did not prevent the growth of *L. monocytogenes* at 4C when incorporated in a food with a pH of 5.2. However, there appears to be no information available on how malic acid concentration influences survival of foodborne pathogens under conditions that do not support growth. Accordingly, the objective of the current study was to determine how pH and malic acid concentration interact to affect the inactivation kinetics of *L. monocytogenes*. The study was conducted in a manner that allowed the results to be compared directly with our previous studies with acetic, lactic, and

MATERIALS AND METHODS

The methods employed in the current study are the same as those employed in our earlier studies (Buchanan and Golden 1994; Buchanan *et al.* 1993). These methods are described briefly below.

Preparation of Cultures

Stock cultures of *L. monocytogenes* (Scott A, HO-VJ-S and V-7) were maintained in brain heart infusion broth (BHI) (Difco, Detroit, MI) at 5C and transferred monthly. Starter cultures of each strain were grown individually in 125-mL Erlenmeyer flasks containing 25-mL BHI + 0.3% dextrose for 24 h at 37C on a rotary shaker (150 rpm). The final pH of the cultures were approximately 5.0, ensuring that the isolates were induced to an acid tolerant state (Farber and Pagotto 1992; Buchanan *et al.* 1994). The three cultures were then combined to achieve approximately equal levels of the three strains (ca. 10^9 cfu/mL).

Preparation of the Test System

Combinations of malic acid and monosodium malate were added to BHI to achieve total concentrations of 0.1, 0.5, 1.0 and 2.0 M with approximately the desired pH. These concentrations are approximately equivalent to 1.3, 6.7, 13.4, and 26.8 % (W/V) malic acid. Final adjustments were made using concentrated HCl or NaOH to achieve pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. The pH of control cultures without malic acid (0.0 M) were adjusted using concentrated HCl. Duplicate 20-mL portions of each of the 45 pH/concentration combinations were transferred to 150-mL milk dilution bottles and sterilized by autoclaving at 121C for 15 min. The pH of the media was verified after autoclaving to insure that it was within 0.1 units of the target value.

Inactivation Studies

Each bottle was inoculated with the 3-strain starter culture to an initial population density of approximately 10^8 cfu/mL. Bottles were stored on their sides to maximize oxygen transfer and held at 28C without agitation. Samples were removed periodically, diluted in 0.1% peptone water, and plated in duplicate on tryptic soy agar (TSA) (Difco) using a spiral plater (Spiral Systems, Inc., Cincinnati, OH). All plates were incubated for 24 h at 37C and enumerated using an automated colony counter (Model 500A, Spiral Systems, Inc.). Sampling

Survivor Curves

Survivor curves were generated by fitting the $\log_{10}$ counts to the linear model of Buchanan *et al.* (1993).

$$Y = Y_0 \quad \text{for } t \leq t_L$$
$$Y = Y_0 + m\,(t - t_L) \quad \text{for } t \geq t_L \qquad (1)$$

Where:

$Y = \log_{10}$ count of bacteria at time t	[$\log_{10}$ (cfu/mL)]
$Y_0 = \log_{10}$ count of bacteria at time t = 0	[$\log_{10}$ (cfu/mL)]
m = slope of the survivor curve	[$\log_{10}$ (cfu/mL)/h]
t = time	[h]
t_L = duration of lag period prior to initiation of inactivation	[h]

The "t_L" and "m" terms were fitted using ABACUS, a curve fitting program (Damert 1994). The Y_0-value was fixed at that observed for the 0-h sample. D-values were calculated as the negative reciprocal of m, and the "$t_{4\text{-}D}$" were calculated using the equation:

$$t_{4\text{-}D} = t_L + (4 \times D) \qquad (2)$$

Undissociated malic acid concentrations were calculated using a pK_a of 3.4 (Weast 1969).

RESULTS AND DISCUSSION

The effect of pH and malic acid concentration on the t_L, D, and $t_{4\text{-}D}$ values associated with the inactivation of *L. monocytogenes* are summarized in Table 1. Lag periods before initiation of inactivation were observed with some pH/acid combinations, particularly at the higher and intermediate pH values. Lag periods complicate the use of inactivation data to predict inactivation rates since reporting only D-values could lead to predicted inactivation kinetics that underestimate the survival of the microorganism. Since our primary interest was predicting holding times needed to produce a substantial inactivation of the pathogen, analysis of the data concentrated on the effects of the variables on $t_{4\text{-}D}$ values, a measurement that

densities prior to the initiation of inactivation (Table 1). As in the earlier studies, this period of growth was included in the overall calculation of inactivation times.

The effect of pH alone was assessed by examining the cultures where pH was adjusted with HCl alone. In the absence of malic acid, decreasing the pH to < 6.0 produced increasingly shorter times to achieve a 4-D inactivation. The inactivation rates were similar to those observed previously, except that the pH 6.0 cultures had substantially longer D-values than in the earlier studies (Buchanan *et al.* 1993; Buchanan and Golden 1994). The reason for this increase in acid resistance at pH 6.0 is not readily apparent since the response at other pH values was reasonably consistent among the different studies. As in the earlier investigations, the relationship between pH and $t_{4\text{-}D}$ was approximately linear at pH values ≤ 5.5 for cultures without added organic acid.

When malic acid was present in the BHI, $t_{4\text{-}D}$ values were dependent on both acidulant concentration and pH (Table 1). At pH values ≥5.5, cultures containing ≤ 1.0 M malic acid had $t_{4\text{-}D}$ values greater than the corresponding control cultures, indicating that the organic acid enhanced the organism's survival. In some cases this effect was quite substantial. For example, the $t_{4\text{-}D}$ values for the pH 5.5 cultures containing 0.1, 0.5, and 1.0 M malic acid were roughly double that of the control cultures. Malic acid affected *L. monocytogenes* inactivation in a manner qualitatively similar to that observed with citric acid (Buchanan and Golden 1994). At pH 7.0 and 6.0, 0.1 and 0.5 M citric acid enhanced the survival of *L. monocytogenes*, while higher concentrations of this tricarboxylic acid accelerated inactivation (Buchanan and Golden 1994). It is important to note that it is only the lower concentrations of malic acid in the current study that would actually be encountered in foods. This protective effect was not observed when lactic or acetic acids were used as acidulants (Buchanan *et al.* 1993). Buchanan and Golden (1994) speculated that the protective effect observed with citric acid may be related to its ability to chelate various trace minerals. Like citric acid, malic acid acts as both an acidulant and a chelating agent in foods. Malate has also been reported to enhance the survival of *Lactobacillus plantacum* in low pH conditions by providing an energy source that is not coupled to H^+ ATPase activity (Garcia *et al.* 1992).

When the pH was <5.5, malic acid produced a dose-dependent enhancement of *L. monocytogenes* inactivation (Table 1). Accelerated inactivation was also observed with the pH 5.5 and 6.0 cultures that contained 2.0 M malic acid. In absolute terms, increasing malic acid concentrations produced its greatest effect at pH 4.0 to pH 5.0 (Fig. 1a). However, when the $t_{4\text{-}D}$ data are plotted on a log scale, it is apparent that the organic acid had its greatest relative effect at pH 3.0 to 4.0 (Fig. 1b). This effect of pH on the absolute and relative impact of acidulant

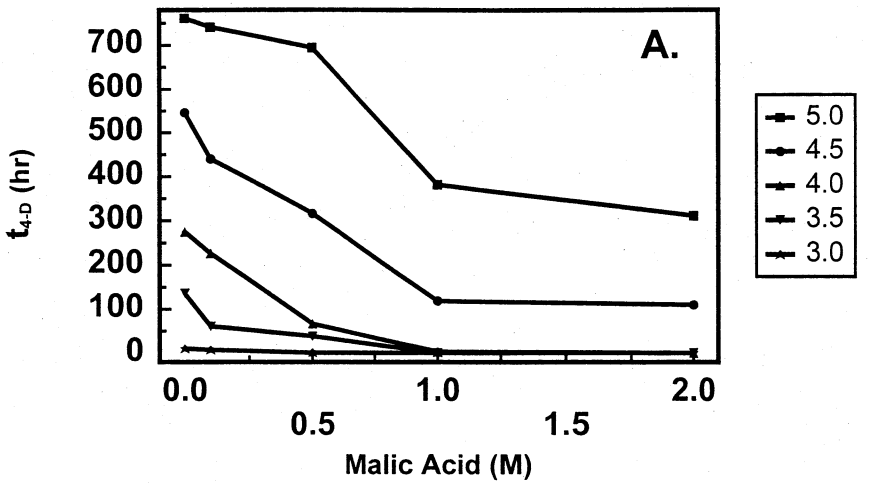
TABLE 1.
EFFECTS OF pH AND MALIC ACID CONCENTRATION ON THE SURVIVAL OF A THREE STRAIN MIXTURE OF *LISTERIA MONOCYTOGENES* IN BRAIN HEART INFUSION AT 28C. VALUES REPRESENT THE MEANS OF DUPLICATE SAMPLES FOR TWO INDEPENDENT TRIALS[a]

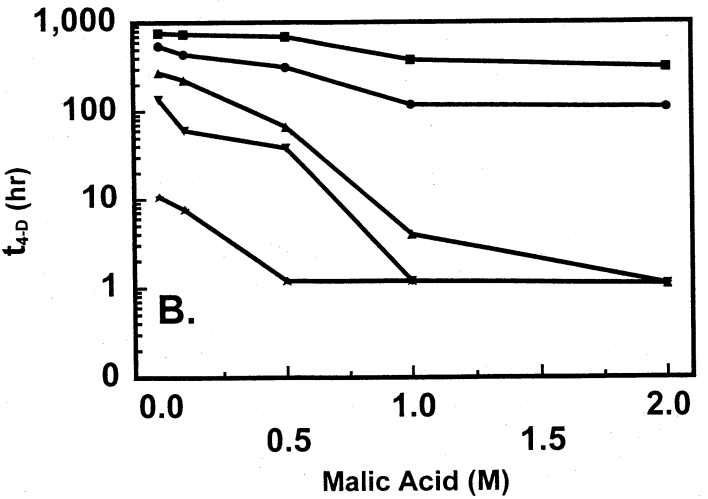
pH	Malic Acid (M)	t_L-Value (h)	D-Value (h)	$t_{4\text{-}D}$-Value** (h)
7.0	0.0	864.0*	692.8	3635.1
	0.1	1176.0*	684.2	3912.9
	0.5	864.0*	791.3	4029.1
	1.0	168.0*	931.4	3893.6
	2.0	168.0	810.6	3410.3
6.5	0.0	504.0*	831.0	3484.4
	0.1	1176.0*	764.3	4233.1
	0.5	168.0*	1114.8	4627.1
	1.0	168.0*	1019.0	4244.1
	2.0	168.0	923.6	3862.4
6.0	0.0	6.0*	869.6	3484.4
	0.1	168.0*	1054.1	4384.2
	0.5	336.0*	1160.1	4976.5
	1.0	384.0*	931.5	4110.1
	2.0	0.0	375.0	1500.2
5.5	0.0	408.0*	276.5	1514.1
	0.1	672.0*	438.3	2425.1
	0.5	480.0*	655.7	3102.7
	1.0	570.7	389.6	2129.1
	2.0	72.0	160.1	712.5
5.0	0.0	411.3	87.6	761.3
	0.1	297.1	111.0	741.2

pH	Malic Acid (M)	t_L-Value (h)	D-Value (h)	$t_{4\text{-}D}$-Value** (h)
4.5	0.0	0.0	118.6	545.3
	0.1	0.0	110.0	439.8
	0.5	6.0	77.5	315.9
	1.0	0.0	29.8	119.1
	2.0	0.0	27.5	110.0
4.0	0.0	52.1	55.8	275.2
	0.1	0.0	56.6	226.4
	0.5	0.0	16.7	66.9
	1.0	0.0	1.0	4.0
	2.0	0.0	0.3	1.1
3.5	0.0	25.3	27.7	136.1
	0.1	0.0	15.3	61.0
	0.5	0.0	9.6	38.4
	1.0	0.0	0.3	1.2
	2.0	0.0	0.3	1.1
3.0	0.0	2.0	2.1	10.6
	0.1	0.0	1.9	7.6
	0.5	0.0	0.3	1.2
	1.0	0.0	0.3	1.2
	2.0	0.0	0.3	1.1

*Population density increased by 2- to 10-fold prior to the initiation of inactivation. This period of growth was included as part of t_L.
**Estimated time to a 99.99 % (4-D) decrease in population density.
[a]The range of individual values was typically within 10% of the mean.

Previously, an empirical linear relationship was identified between the logarithm of the t_L values and the square root of the concentration of


A.
700
600
500
400
300
200
100
0
$t_{4\text{-}D}$ (hr)
0.0
0.5
1.0
1.5
2.0
Malic Acid (M)
5.0
4.5
4.0
3.5
3.0


1,000
100
10
1
0
B.
$t_{4\text{-}D}$ (hr)
0.0
0.5
1.0
1.5
2.0
Malic Acid (M)

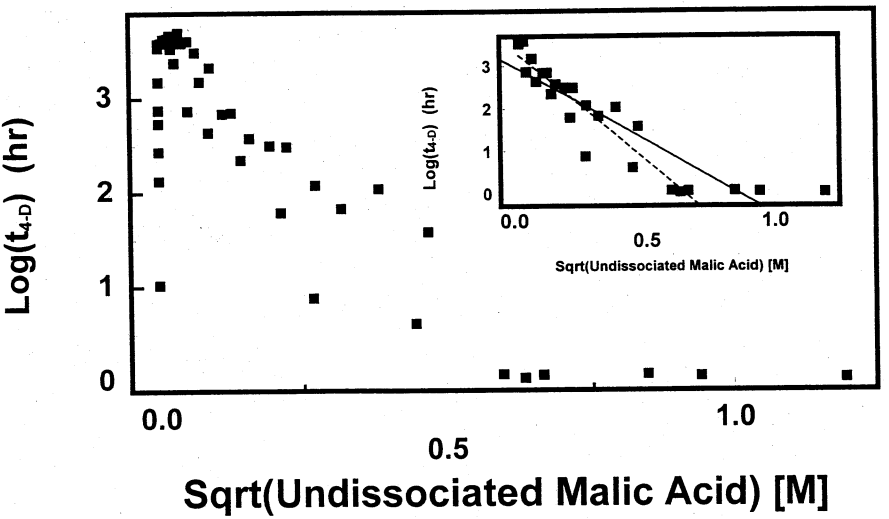


FIG. 2. THE RELATIONSHIP BETWEEN THE LOG (t_{4-D}) AND THE SQUARE ROOT OF THE CALCULATED CONCENTRATIONS OF UNDISSOCIATED MALIC ACID
The insert is the same data where the log (t_{4-D}) for selected variable combinations are excluded (see text). The linear regression lines for these data with (—) and without (---) the three highest concentrations of undissociated malic acid are depicted in the insert.

evident, though the relationship was not as strong as previously observed with lactic, acetic, and citric acids. However, the R^2 value increased to 0.870 when the extremely rapid inactivation values at the three highest undissociated malic acid concentrations were excluded from the analysis. The relationship under those conditions was described by the equation:

$$\text{Log}(t_{4-D}) = -5.1545[\text{HA}]^{0.5} + 3.3639$$

This relationship supports the hypothesis that, like other organic acids, the anion effects observed with malic acid are related to it being present in its completely undissociated form. Buchanan *et al.* (1997) compared the empirical relationship between $\log(t_{4-D})$ and $[\text{HA}]^{0.5}$ for lactic acid with a more detailed

The results of the current study indicate that while malic acid and pH can interact to enhance the inactivation of *L. monocytogenes* in acidic environments, this acid is one of the more benign in relation to anion effects (Buchanan *et al.* 1993; Buchanan and Golden 1994). This is in agreement with the results of the few studies that have examined the effectiveness of malic acid to control *L. monocytogenes*. Sorrells *et al.* (1989) reported that microbiological media acidified to pH 4.4 with malic acid supported the growth of *L. monocytogenes* at 10, 25, and 35C. Glass *et al.* (1995) found that using malic acid as the acidulant for curd formation did not prevent the growth of *L. monocytogenes* in queso blanco style cheese at either 4 or 20C. Malic acid also appears to have a similar impact on enterohemorrhagic *Escherichia coli.* A mixture of three O157:H7 isolates grew at 25C in media adjusted to pH 4.0 with malic acid (final concentration = 0.039 M), and survived for >28 days before any decline in surviving populations were observed (Conner and Kotrola 1995). This is also in keeping with the observations that *E. coli* O157:H7 can survive for extended periods in apple cider or apple juice, particularly at refrigeration temperatures (Zhao *et al.* 1993; Miller and Kaspar 1994; Leyer *et al.* 1995). The concentration of malic acid in apple juice generally ranges from 0.3 to 1.0% depending on factors such as variety, maturity, and storage conditions (Hulme and Rhodes 1971). Overall, the antimicrobial activity characteristics of malic acid are similar to those of citric acid. The relative gentleness of these organic acids in combination with refrigerated storage are likely factors that contributed to recent outbreaks of foodborne disease associated with fresh fruit juices.

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